

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE *l*IVE GENE

[0001] BACKGROUND OF THE INVENTION

The invention provides nucleotide sequences from coryneform bacteria which code for the *ilvE* gene and a process for the fermentative preparation of amino acids using bacteria in which the endogenous *ilvE* gene is enhanced. All references cited herein are expressly incorporated by reference. Incorporation by reference is also designated by the term "*I.B.R.*" following any citation.

[0002] L-Amino acids, in particular L-leucine, L-valine, L-isoleucine and L-phenylalanine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

[0003] It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

[0005] Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

[0006] The invention provides new measures for improved fermentative preparation of amino acids.

[0007] BRIEF SUMMARY OF THE INVENTION

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-leucine, L-valine, L-isoleucine and L-phenylalanine are particularly preferred.

[0008] The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the *ilvE* gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of transaminase E.

[0009] The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence shown in SEQ ID No. 1, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i) which do not modify the activity of the protein/polypeptide.

[0010] Finally, the invention also provides polynucleotides chosen from the group consisting of

- a) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1 and 220,
- b) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 221 and 1324,
- c) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1325 and 1453.

[0011] The invention also provides

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid vector, and

coryneform bacteria which contain the vector or in which the endogenous ilvE gene is enhanced.

[0012] The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

[0013] DETAILED DESCRIPTION OF THE INVENTION

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for transaminase E or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence with the sequence of the ilvE gene. They are also suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides

[0014] Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for transaminase E can be prepared by the polymerase chain reaction (PCR).

[0015] Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40, or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

[0016] "Isolated" means separated out of its natural environment.

[0017] "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

[0018] The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least in particular 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

[0019] "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

[0020] The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of transaminase E, and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

[0021] The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences which code for the *ilvE* gene are enhanced, in particular over-expressed.

[0022] The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

[0023] By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

[0024] The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose,

lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

[0025] Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

[0026] The new *ilvE* gene from *C. glutamicum* which codes for the enzyme transaminase E (EC 2.6.1.42) has been isolated.

[0027] To isolate the *ilvE* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie [Genes and Clones, An Introduction to Genetic Engineering] (Verlag Chemie, Weinheim, Germany, 1990) *I.B.R.*, or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) *I.B.R.* may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by

Kohara et al. (Cell 50, 495 -508 (1987)) I.B.R.. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) I.B.R. describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164 I.B.R.) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575 I.B.R.).

[0028] Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) I.B.R. in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980) I.B.R.).

[0029] To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979) I.B.R.) or pUC9 (Vieira et al., 1982, Gene, 19:259-268 I.B.R.). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) I.B.R. The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977) I.B.R.

[0030] The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)) I.B.R., that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) I.B.R. or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)) I.B.R.

[0031] The new DNA sequence of *C. glutamicum* which codes for the *ilvE* gene and which, as SEQ ID No. 1, is a

constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the *ilvE* gene product is shown in SEQ ID No. 2. It is known that enzymes endogenous in the host can split off the N-terminal amino acid methionine or formylmethionine of the protein formed.

[0032] Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. Such mutations are also called, *inter alia*, neutral substitutions. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, *inter alia*, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)) *I.B.R.*, in O'Regan et al. (Gene 77:237-251 (1989)) *I.B.R.*, in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)) *I.B.R.*, in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) *I.B.R.* and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

[0033] In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the

invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

[0034] Instructions for identifying DNA sequences by means of hybridization can be found by the expert, *inter alia*, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) *I.B.R.* and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260) *I.B.R.* The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996 *I.B.R.*).

[0035] A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995 I.B.R.) a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. Further instructions

on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

[0036] Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, *inter alia*, in the handbook by Gait: *Oligonucleotide Synthesis: A Practical Approach* (IRL Press, Oxford, UK, 1984) *I.B.R.* and in Newton and Graham: *PCR* (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994) *I.B.R.*

[0037] It has been found that coryneform bacteria produce amino acids in an improved manner after over-expression of the *ilvE* gene.

[0038] To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative amino acid production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

[0039] Instructions in this context can be found by the expert, *inter alia*, in Martin et al. (*Bio/Technology* 5, 137-146 (1987)) *I.B.R.*, in Guerrero et al. (*Gene* 138, 35-41 (1994)) *I.B.R.*, Tsuchiya and Morinaga (*Bio/Technology* 6,

428-430 (1988)) *I.B.R.*, in Eikmanns et al. (Gene 102, 93-98 (1991)) *I.B.R.*, in European Patent Specification 0 472 869 *I.B.R.*, in US Patent 4,601,893 *I.B.R.*, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) *I.B.R.*, in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) *I.B.R.*, in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)) *I.B.R.*, in Patent Application WO 96/15246 *I.B.R.*, in Malumbres et al. (Gene 134, 15 - 24 (1993)) *I.B.R.*, in Japanese Laid-Open Specification JP-A-10-229891 *I.B.R.*, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) *I.B.R.*, in Makrides (Microbiological Reviews 60:512-538 (1996)) *I.B.R.* and in known textbooks of genetics and molecular biology.

[0040] By way of example, for enhancement the *ilvE* gene according to the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554 *I.B.R.*), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991) *I.B.R.*) or pHs2-1 (Sonnen et al., Gene 107:69-74 (1991) *I.B.R.*) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160 *I.B.R.*), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990) *I.B.R.*), or pAG1 (US-A 5,158,891 *I.B.R.*), can be used in the same manner.

[0041] Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) *I.B.R.* for duplication or amplification of the *hom-thrB* operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are,

for example, pSUP301 (Simon et al., *Bio/Technology* 1, 784-791 (1983) *I.B.R.*), pK18mob or pK19mob (Schäfer et al., *Gene* 145, 69-73 (1994) *I.B.R.*), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). *Journal of Biological Chemistry* 269:32678-84 *I.B.R.*; US-A 5,487,993 *I.B.R.*), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., *Journal of Molecular Biology*, 234: 534-541 (1993) *I.B.R.*), pEM1 (Schrumpf et al, 1991, *Journal of Bacteriology* 173:4510-4516 *I.B.R.*) or pBGS8 (Spratt et al., 1986, *Gene* 41: 337-342 *I.B.R.*). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (*Applied and Environmental Microbiology* 60, 756-759 (1994)) *I.B.R.* Methods for transformation are described, for example, by Thierbach et al. (*Applied Microbiology and Biotechnology* 29, 356-362 (1988) *I.B.R.*), Dunican and Shivnan (*Bio/Technology* 7, 1067-1070 (1989) *I.B.R.*) and Tauch et al. (FEMS *Microbiological Letters* 123, 343-347 (1994)) *I.B.R.* After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

[0042] In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the *ilvE* gene.

[0043] Thus, for the preparation of L-amino acids, in addition to enhancement of the *ilvE* gene, one or more endogenous genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), *Journal of Bacteriology* 174:6076-6086 *I.B.R.*),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), *Journal of Bacteriology* 174:6076-6086 *I.B.R.*),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), *Journal of Bacteriology* 174:6076-6086 *I.B.R.*),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661 *I.B.R.*),
- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., *European Journal of Biochemistry* 254, 395-403 (1998) *I.B.R.*),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512),
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171 *I.B.R.*),
- the ilvA gene which codes for threonine dehydratase (Möckel et al., *Journal of Bacteriology* (1992) 8065-8072 *I.B.R.*) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) *Molecular Microbiology* 13: 833-842 *I.B.R.*),
- the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739 *I.B.R.*),
- the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) *Applied and Environmental Microbiology* 65: 1973-1979 *I.B.R.*),

- the leuB gene which codes for 3-isopropyl malate dehydrogenase (Patek et al. (1998) Applied and Microbiology Biotechnologie 50: 42-47 *I.B.R.*)
- the brnE gene which codes for valine export (DE: 19951708.8 *I.B.R.*)
- the leuA gene which codes for isopropyl malate synthase (Patek et al. (1994) Applied and Environmental Microbiology 60 (1): 133-140 *I.B.R.*)
- the pyc gene which codes for pyruvate carboxylase (Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998) *I.B.R.*)
- the zwal gene which codes for the Zwal protein (DE: 19959328.0 *I.B.R.*, DSM 13115),

can be enhanced, in particular over-expressed.

[0044] It may furthermore be advantageous for the production of L-amino acids, in addition to the enhancement of the ilvE gene, for one or more of the genes chosen from the group consisting of:

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1 *I.B.R.*; DSM 13047),
- the dapA gene which codes for dihydروdipicolinate synthase (EP-B 0 197 335 *I.B.R.*),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478 *I.B.R.*; DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7 *I.B.R.*; DSM 13114),
- the zwal gene which codes for the Zwal protein (DE: 19959327.2 *I.B.R.*, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

[0045] In addition to over-expression of the *ilvE* gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982 *I.B.R.*).

[0046] The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology] (Gustav Fischer Verlag, Stuttgart, 1991) *I.B.R.*) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994) *I.B.R.*).

[0047] The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981 *I.B.R.*).

[0048] Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic

acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

[0049] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

[0050] Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

[0051] Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired

product has formed. This target is usually reached within 10 hours to 160 hours.

[0052] Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190 *I.B.R.*) by ion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174 *I.B.R.*).

[0053] The process according to the invention is used for fermentative preparation of amino acids.

[0054] The present invention is explained in more detail in the following with the aid of embodiment examples.

[0055] The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA *I.B.R.*). Methods for transformation of *Escherichia coli* are also described in this handbook.

[0056] The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

[0057] Example 1

Preparation of a genomic cosmid gene library from *Corynebacterium glutamicum* ATCC 13032

[0058] Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 is isolated as described by Tauch et al. (1995, Plasmid 33:168-179 *I.B.R.*) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02

I.B.R.). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) *Proceedings of the National Academy of Sciences USA* 84:2160-2164 *I.B.R.*), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) is cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02 *I.B.R.*) and likewise dephosphorylated with shrimp alkaline phosphatase.

[0059] The cosmid DNA is then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04 *I.B.R.*). The cosmid DNA treated in this manner is mixed with the treated ATCC13032 DNA and the batch is treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04 *I.B.R.*). The ligation mixture is then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217 *I.B.R.*).

[0060] For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, *Nucleic Acid Research* 16:1563-1575 *I.B.R.*) the cells are taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor) *I.B.R.*, the cells being plated out on LB agar (Lennox, 1955, *Virology*, 1:190 *I.B.R.*) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones are selected.

[0061] Example 2

Isolation and sequencing of the *ilvE* gene

[0062] The cosmid DNA of an individual colony is isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp are isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0063] The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), is cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04 *I.B.R.*). The ligation of the cosmid fragments in the sequencing vector pZero-1 is carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor *I.B.R.*), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany *I.B.R.*). This ligation mixture is then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7 *I.B.R.*) into the *E. coli* strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 mg/l zeocin.

[0064] The plasmid preparation of the recombinant clones is carried out with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing is carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) *I.B.R.* with modifications according to Zimmermann et al. (1990, Nucleic Acids Research,

18:1067) *I.B.R.* The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany *I.B.R.*) was used. The separation by gel electrophoresis and analysis of the sequencing reaction are carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

[0065] The raw sequence data obtained are then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231 *I.B.R.*) version 97-0. The individual sequences of the pZero1 derivatives are assembled to a continuous contig. Further analyses can be carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402 *I.B.R.*) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA) *I.B.R.*

[0066] The relative degree of substitution or mutation in the polynucleotide or amino acid sequence to produce a desired percentage of sequence identity can be established or determined by well-known methods of sequence analysis. These methods are disclosed and demonstrated in Bishop, et al. "DNA & Protein Sequence Analysis (A Practical Approach)", Oxford Univ. Press, Inc. (1997) *I.B.R.* and by Steinberg, Michael "Protein Structure Prediction" (A Practical Approach), Oxford Univ. Press, Inc. (1997) *I.B.R.*

[0067] The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence shows an open reading frame of 1103 base pairs, which is called the *ilvE* gene. The *ilvE* gene codes for a protein of 367 amino acids.

[0068] This application claims priority to German Priority Document Application No. 100 63 314.5, filed on December

20, 2000. The above German Priority Document is hereby incorporated by reference in its entirety.